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**Title:** Obesity and Postmenopausal Breast Cancer Risk: Determining the Role of Growth Factor-Induced Aromatase Expression

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14. ABSTRACT In postmenopausal women, obesity is associated with a worse breast cancer prognosis, and this may be related to these patients' reduced response rate to aromatase inhibitor treatment. Obesity is accompanied by elevated levels of growth factors and inflammatory cytokines that can promote tumorigenesis and regulate the expression and activity of aromatase, the key enzyme in the synthesis of estrogen from androgens. The purpose of this project is to elucidate the role of local aromatase expression and activity in the promotion of breast cancer progression in obese postmenopausal women. Results so far have demonstrated that serum from obese postmenopausal women stimulates greater aromatase expression in adipose stromal cells (ASC) and MCF-7 breast cancer cells via direct and indirect mechanisms in comparison to serum from normal weight subjects. The obesity-induced aromatase expression in ASC and MCF-7 cells is associated with elevated estrogen receptor alpha activity in the presence of exogenous testosterone. Further data indicates that the indirect effects of the obese sera on ASC aromatase expression are primarily mediated by inflammatory cytokine-induced prostaglandin E2 production by the cancer cells and macrophages. Through identification of these mediators, we hope to develop a more effective chemotherapeutic regimen for the obese postmenopausal population.					
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## **Introduction:**

In postmenopausal women, epidemiological studies have shown that obesity increases the risk of developing breast cancer by approximately 40% (1, 2). It has also been established that obesity is associated with a worse breast cancer prognosis for both pre- and postmenopausal women, with the most significant effects seen in postmenopausal, estrogen receptor alpha (ER $\alpha$ ) positive breast cancer (3-6). Following menopause, adipose tissue becomes the primary site of estrogen synthesis, which is catalyzed by the enzyme aromatase (7). Consequently, obese postmenopausal women have, on average, higher levels of circulating estrogens, leading to the speculation that elevated estrogen synthesis by the adipose tissue may be the primary mediator of breast tumorigenesis in this population (8). However, aromatization of androgens into estrogen also occurs in both the epithelial and adipose components of breast tissue, and examination of breast carcinoma tissue samples has revealed estrogen concentrations ten-fold greater than circulating plasma levels and two times the concentration found in normal breast tissue. This is correlated with a four to five-fold increase in aromatase expression within the breast tumor and tumor-bearing adipose quadrants (9). Thus, if increased estrogen levels are indeed driving greater breast cancer progression in obese postmenopausal women, it seems more likely that the key source of estrogen is local production within the mammary tissue, which obesity may be promoting. A recent study from Morris et al (10) supports this theory by showing that aromatase levels in the mammary tissue of breast cancer patients correlate with body mass index (BMI).

In addition to an increase in circulating estrogens, obesity is also associated with elevated levels of inflammatory cytokines and growth factors, and both are known to play a role in breast tumorigenesis (11-20). Specifically, the serum concentration of interleukin 6 (IL-6) is increased with obesity, and this inflammatory cytokine can promote aromatase expression both directly and indirectly in breast cancer epithelial cells and adipose stromal cells (ASC), the primary site of aromatase expression within the adipose tissue (21). Indirectly, IL-6 can enhance aromatase expression by promoting COX-2 expression in cancer epithelial cells and thereby inducing local production of prostaglandin E2 (PGE2), a potent stimulator of aromatase expression (21-22). Free insulin-like growth factor 1 (IGF-1), which is increased with obesity, may also be playing a role. This growth factor can regulate the expression and activity of aromatase, particularly in breast cancer epithelial cells (23-25). Taken together, this data led us to hypothesize that obesity-associated circulating factors may be driving local aromatase expression and activity even higher, thereby promoting greater local estrogen production and activity. The purpose of this project is to elucidate the role of local aromatase expression and activity in the promotion of breast cancer progression in obese postmenopausal women.

## **Body:**

***Task 1:** Complete planned coursework that provides background and specific knowledge necessary for the PI's development as a breast cancer researcher with an emphasis on the role of nutrition (months 1-8).*

All courses have been completed by the PI.

***Task 2:** Attend and present data at national conferences to enhance the PI's presentation skills, publicize her work, and provide opportunities for networking and discussion with other researchers regarding their work (months 1-36).*

The PI presented posters at the 2012 AACR annual conference (Task 2d) and a 2012 Keystone Symposium on "The Role of Inflammation during Carcinogenesis" (Task 2c) (see Appendix B for abstracts). The PI attended the 2012 CTCR-AACR San Antonio Breast Cancer Research Symposium, but did not present a poster (Task 2e).

***Task 3:** Participate in the weekly seminar series hosted by the Department of Nutritional Sciences at UT Austin and the University of Texas MD Anderson Cancer Center-Science Park, which includes presentations by researchers from other institutions, departmental faculty, and departmental graduate students (months 1-36).*

The weekly department seminar series was attended (Task 3a), and the PI presented her cumulative research findings at seminars in April 2012 and January 2013 (Task 3b).

Task 4: Complete animal study for Specific Aim 1A (months 1-36).

Task 4a has been completed. As of the end of the reporting period for this annual summary (12-14-12), Tasks 4b and 4c had been completed for a total of 40 mice, 20 in the 6-month diet group and 20 in the 4-month diet group. Tasks 4d and 4f have been performed on these mice according to the specified schedule. Twenty mice have completed the study, with the tissues and sera collected according to Tasks 4h and 4i. Within the 6-month diet group, 3 mice in the diet-induced obesity (DIO) group and 3 in the control group have developed tumors; both were euthanized prior to completion of the diet time period due to tumor size  $>1.5 \text{ cm}^2$ . Within the 4-month diet group, 2 DIO mice and 1 control mouse have developed tumors and been euthanized. While there is currently no difference in tumor incidence between the DIO and control groups, preliminary data suggests that the tumors are growing more quickly in the DIO group. The average time between the identification of a palpable tumor and growth to  $>1.5 \text{ cm}^2$  is eight days for the DIO group versus 16 days for the controls. The PI has received training on qPCR, but not IHC technique (Task 4j). Molecular analysis of the sera and tissues collected from the mice (Tasks 4k-n) and DEXA analysis of the mouse carcasses (Task 4e) will occur according to the timeline specified in the revised statement of work (SOW).

Task 5: Complete in vitro studies for Specific Aim 1B (months 13-36).

The *in vitro* studies for Specific Aim 1B are in progress. Tasks 5a, 5b, 5c, and 5h have been completed. Key findings so far include (see Appendix A for figures):

Task 5e:

- Figure 1: Ob serum stimulated greater Akt and ERK1/2 phosphorylation in MCF-7 and T47D cells after 15 minute and 1 hour exposures in comparison to Con serum ( $p<0.05$ ).
- Figure 2: A 15 minute and 1 hour exposure to Ob serum stimulated greater ER $\alpha$  phosphorylation at ser167 (the Akt target site) ( $p<0.05$ ), but not ser118 (the Erk1/2 target site), in MCF-7 cells vs Con sera.
- Figure 3: A 1 hour exposure to Ob serum did not promote more or less total ER $\beta$  expression in MCF-7 or ZR75 cells vs Con sera.

Tasks 5g and 5j:

- Figure 4: MCF-7 cells exposed to Ob serum for 24 hours had 41% greater aromatase expression, as measured by qPCR, in comparison to Con sera ( $p<0.05$ ). The Ob serum-induced MCF-7 cell aromatase expression was significantly inhibited by the Akt II inhibitor and PD98059 (the MAPK inhibitor), but not LY294002 (the PI3K inhibitor). However, these same drug treatments all significantly increased Con serum-induced aromatase expression in the MCF-7 cells, possibly due to inhibition of negative feedback loops ( $p<0.05$ ).
- Figure 5: Conditioned media (CM) from ASC exposed to Ob serum promoted 72% greater MCF-7 cell aromatase expression in comparison to CM from ASC exposed to Con serum ( $p<0.05$ ). ASC CM was generated by exposing ASC to Ob or Con human serum for 3 hours, removing the serum and washing the cells with PBS, then incubating the ASC in serum-free media (SFM) for 24 hours. The SFM (now CM) was then collected and either used immediately to treat MCF-7 cells or frozen at  $-20^\circ\text{C}$  for later use. The MCF-7 cells were exposed to the ASC CM for 24 hours. Neutralization of IL-6 in both the Ob and Con ASC CM (via the addition of an IL-6 antibody to the CM for 1 hour prior to its use as a treatment for the MCF-7 cells) significantly decreased the CM-induced MCF-7 cell aromatase expression, eliminating the difference between Ob and Con ( $p<0.05$ ).
- Figure 6: There was no significant difference in the aromatase expression in ASC exposed to Ob serum vs Con for 24 hours.

- Figure 7A: CM from MCF-7 cells exposed to Ob serum induced 65% greater aromatase expression in ASC in comparison to CM from MCF-7 cells exposed to Con serum ( $p<0.05$ ). MCF-7 cell CM was generated by exposing MCF-7 cells to Ob or Con human serum for 1 hour, removing the serum and washing the cells with PBS, then incubating the MCF-7 cells in SFM for 24 hours. The SFM (now CM) was then collected and either used immediately to treat the ASC or frozen at  $-20^{\circ}\text{C}$  for later use. The ASC were exposed to the MCF-7 CM for 24 hours. Inhibition of COX-2 activity in MCF-7 cells using celecoxib (30  $\mu\text{M}$ ) during the generation of CM with Ob and Con sera significantly reduced Ob and Con CM-induced ASC aromatase expression, eliminating the difference between Ob and Con ( $p<0.05$ ). Neutralization of IL-6 in both the Ob and Con sera (via the addition of an IL-6 antibody to the sera for 1 hour prior to its use to generate CM) also significantly decreased the CM-induced ASC aromatase expression, again eliminating the difference between Ob and Con ( $p<0.05$ ).
- Figure 7B: Ob MCF-7 CM that was generated as stated above contained approximately 13 times more PGE2 (as measured by ELISA) in comparison to Con MCF-7 CM ( $p=0.06$ ), supporting our hypothesis that exposure to Ob sera enhances MCF-7 cell COX-2 activity in comparison to Con sera.
- Figure 8: CM from U937 cells (matured from monocytes to macrophages with a 48 hour treatment of 10 ng/ml TPA) exposed to Ob serum induced 52% greater aromatase expression in ASC in comparison to CM from U937 cells exposed to Con serum ( $p<0.05$ ). U937 cell CM was generated by exposing U937 cells to Ob or Con human serum for 1 hour, removing the serum and washing the cells with PBS, then incubating the U937 cells in SFM for 24 hours. The SFM (now CM) was then collected and either used immediately to treat the ASC or frozen at  $-20^{\circ}\text{C}$  for later use. The ASC were exposed to the U937 CM for 24 hours. Inhibition of COX-2 activity in U937 cells with celecoxib (30  $\mu\text{M}$ ) during the generation of CM with Ob and Con sera significantly reduced Ob and Con CM-induced ASC aromatase expression, eliminating the difference between Ob and Con ( $p<0.05$ ).
- Figure 9: ASC exposed to Ob or Con CM from MCF-7 or U937 cells express at least 100-fold more aromatase in comparison to MCF-7 cells exposed to Ob or Con sera or CM from ASC, suggesting that local ASC in the breast may be the more biologically relevant source of aromatase versus the cancer epithelial cells.

Task 6: Complete tissue analysis and in vitro studies for Specific Aim 2, using the same panel of cell lines for the in vitro studies as listed for Aim 1B (months 9-36).

The *in vitro* studies for Specific Aim 2 have also been initiated, but not the tissue analysis (Task 6a) since the mouse study is still ongoing. Key findings so far include (see Appendix A for figures):

Tasks 6c and 6f:

- Figure 10A: MCF-7 and T47D cells exposed to Ob human sera for 24 hours did not demonstrate greater ER $\alpha$  activity in comparison to Con sera, as measured by qPCR analysis of pS2 expression. This indicates that any differences in circulating estrogen levels between obese and control subjects are not sufficient to induce a difference in breast cancer cell ER $\alpha$  activity.
- Figure 10B: MCF-7 and T47D cells exposed to Ob human sera for 24 hours did express 34% and 30% higher levels of cyclin D1, respectively, as measured by qPCR ( $p<0.05$ ). This is likely due to estrogen-independent effects from other circulating factors that are increased in the Ob sera.
- Figure 11: CM from a co-culture of MCF-7 cells and ASC exposed to Ob sera, then SFM with testosterone, promotes greater ER $\alpha$  activity (measured by qPCR analysis of pS2 expression) in separate MCF-7 cells. MCF-7 cells were seeded on top of ASC, then the co-culture was exposed to Ob or Con sera for 1 hour. The sera was then removed and the cells washed with PBS, then incubated in SFM with testosterone (100 nM) +/- anastrozole (1  $\mu\text{M}$ ) for 24 hours. The media (now CM) was then collected and either used immediately to treat MCF-7 cells that had been seeded separately or frozen at  $-20^{\circ}\text{C}$  for later use. The MCF-7 cells were exposed to the ASC-MCF7 CM for 24 hours. This experiment tests whether the greater

ASC aromatase expression induced by exposure to Ob MCF-7 CM versus Con (Figure 7A) then results in greater estradiol production that stimulates more MCF-7 cell ER $\alpha$  activity. The difference seen between Ob and Con here is neutralized by the addition of anastrozole with the testosterone. This experiment has only been completed once, so statistics are not yet available.

- Figure 12: CM from a co-culture of U937 macrophages and ASC exposed to Ob sera, then SFM with testosterone, promotes greater ER $\alpha$  activity (measured by qPCR analysis of pS2 expression) in MCF-7 cells. U937 cells were seeded on top of ASC and incubated in TPA (1 ng/ml) for 48 hours to differentiate the U937 monocytes to macrophages, then the co-culture was exposed to Ob or Con sera for 1 hour. The sera was then removed and the cells washed with PBS, then incubated in SFM with testosterone (100 nM) +/- anastrozole (1  $\mu$ M) for 24 hours. The SFM (now CM) was then collected and either used immediately to treat MCF-7 cells or frozen at -20°C for later use. The MCF-7 cells were exposed to the ASC-U937 CM for 24 hours. This experiment tests whether the greater ASC aromatase expression induced by exposure to Ob macrophage CM versus Con (Figure 8) then results in greater estradiol production that stimulates more MCF-7 cell ER $\alpha$  activity. The difference seen between Ob and Con here is neutralized by the addition of anastrozole with the testosterone ( $p < 0.05$ ).

Tasks 6e and 6f:

- Figure 13A: MCF-7 and T47D cells exposed to Ob human sera for 48 hours did not demonstrate greater ER $\alpha$  activity in comparison to Con sera, as measured by ERE luciferase assay. This indicates that any differences in circulating estrogen levels between obese and control subjects are not sufficient to induce a difference in breast cancer cell ER $\alpha$  activity.
- Figure 13B: There was still no difference in MCF-7 cell ER $\alpha$  activity, as measured by ERE luciferase, when subjects that were on aromatase inhibitor treatment at the time of sera collection were eliminated from the Ob and Con sera pools used to treat the cells for 48 hours. These results indicate that the lack of difference in breast cancer cell ER $\alpha$  activity following Ob versus Con sera exposure is not due to the use of aromatase inhibitor treatment by some subjects.
- Figure 14: When exogenous testosterone (100 nM) is added along with the sera to serve as the aromatase substrate, ER $\alpha$  activity is elevated in the MCF-7 cells exposed to Ob sera for 48 hours, as measured by ERE luciferase. The simultaneous addition of the aromatase inhibitor anastrozole (1  $\mu$ M) significantly decreased the ER $\alpha$  activity induced by Ob sera plus testosterone ( $p < 0.05$ ). These results indicate that the disparity in the aromatase expression (Figure 4) and subsequent estradiol production stimulated by Ob versus Con sera in these cells is large enough to promote a difference in ER $\alpha$  activity.
- Figure 15: CM from ASC exposed to Ob sera (versus Con), which was generated as described for Figure 5, induced greater MCF-7 cell ER $\alpha$  activity in the presence of exogenous testosterone (100 nM). ER $\alpha$  activity in the MCF-7 cells was measured by ERE luciferase following a 48 hour exposure to the ASC CM. The addition of anastrozole (1  $\mu$ M) reduced ER $\alpha$  activity levels back to those seen with Ob CM alone ( $p < 0.05$ ). These results indicate that the disparity in the aromatase expression (Figure 5) and subsequent estradiol production stimulated by Ob versus Con ASC CM in these cells is large enough to promote a difference in ER $\alpha$  activity.

#### **Key Research Accomplishments:**

- Aromatase expression is elevated in MCF-7 cells exposed to Ob human sera and Ob CM from ASC in comparison to controls.
- This obesity-induced increase in MCF-7 cell aromatase expression results in greater MCF-7 cell ER $\alpha$  activity in the presence of exogenous testosterone, an effect that is decreased by the addition of an aromatase inhibitor.

- Aromatase expression is higher in ASC exposed to Ob CM from MCF-7 cells and U937 macrophages in comparison to controls; these effects are likely mediated by increased PGE<sub>2</sub> production by both the MCF-7 cells and macrophages, as treatment of these cells with the COX-2 inhibitor celecoxib during the generation of CM neutralizes the difference in ASC aromatase expression between Ob and Con.
- This obesity-induced increase in ASC aromatase expression results in greater MCF-7 cell ER $\alpha$  activity in the presence of exogenous testosterone, an effect that is neutralized by the addition of an aromatase inhibitor.

### **Reportable Outcomes:**

Conference posters:

*Bowers LW, Li R, Tekmal RR, Brenner AJ, Hursting SD, deGraffenried LA.* Obesity promotes elevated aromatase expression in the breast tumor microenvironment via upregulation of prostaglandin E<sub>2</sub> production. Keystone Symposia: The Role of Inflammation during Carcinogenesis, Dublin, Ireland, 2012

*Bowers LW, De Angel R, Brenner AJ, Tekmal RR, Hursting SD, deGraffenried LA.* Obesity-associated growth factor signaling promotes aromatase expression and estrogen receptor activity in breast cancer cells. 102nd Annual Meeting of the American Association for Cancer Research, Chicago, IL, 2012.

### **Conclusion:**

The results of this project so far indicate that circulating factors associated with obesity do promote greater aromatase expression in breast cancer cells and ASC via direct and indirect actions on the cells. The indirect mechanisms occur through paracrine interactions between these two cell types and between the ASC and macrophages. The obesity-induced elevation in aromatase expression is associated with an increase in ER $\alpha$  activity in the cancer cells. The data indicates that the relative aromatase expression levels are substantially higher in the ASC versus the cancer cells. Consequently, the PI plans to primarily focus on further exploring the pathways responsible for the obesity-induced ASC expression over the next few months. If the inhibition of COX-2 successfully suppresses obesity-induced aromatase expression and breast cancer cell ER $\alpha$  activity, these findings will pave the way for further animal and human studies. The ultimate goal of this project is the identification of a potentially more effective chemotherapeutic regimen for the obese postmenopausal patient population.

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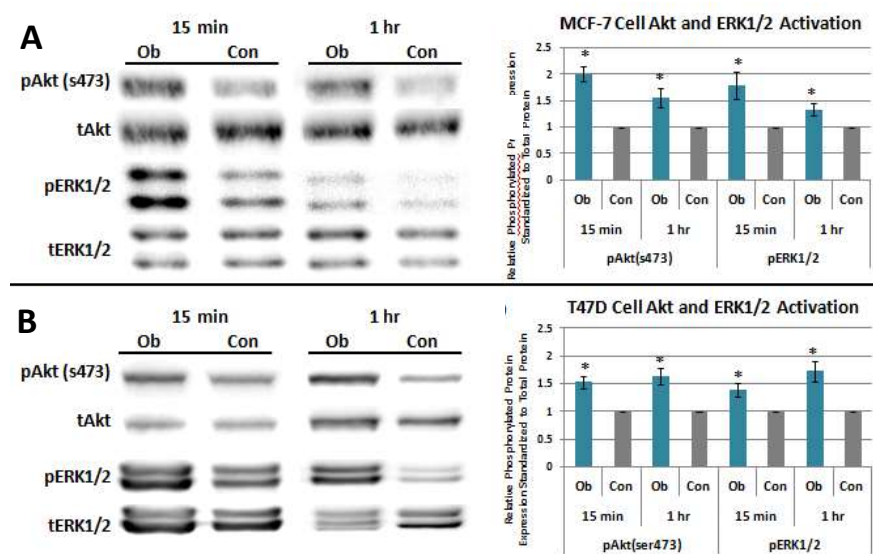
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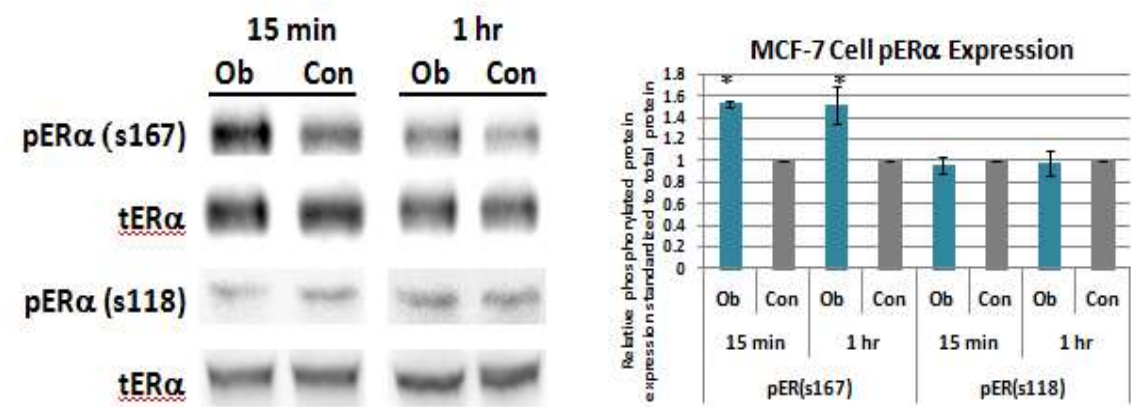
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**Appendix A: Figures**

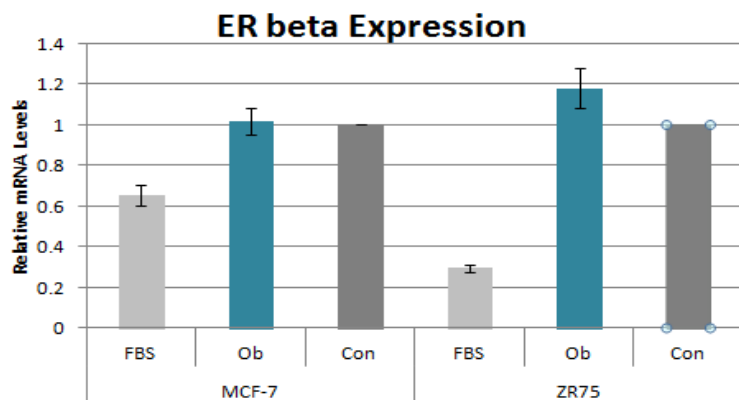
**Figure 1.** Akt (ser473) and ERK1/2 phosphorylation in MCF-7 (A) and T47D (B) cells after 15 minute and 1 hour of Ob or Con human sera exposure. Graphs represent pAkt (ser473) and pERK1/2 protein levels standardized to tAkt and tERK1/2 protein levels, respectively. Asterisks represent significant differences in comparison to Con (p<0.05).



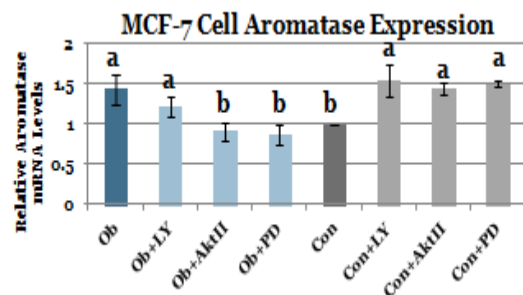
**Figure 2.** ERα phosphorylation in MCF-7 cells at ser167 (the Akt target site) and ser118 (the MAPK target site) after 15 minute and 1 hour of Ob or Con human sera exposure. Graphs represent pERα protein levels standardized to tERα protein levels. Asterisks represent significant differences in comparison to Con (p<0.05).



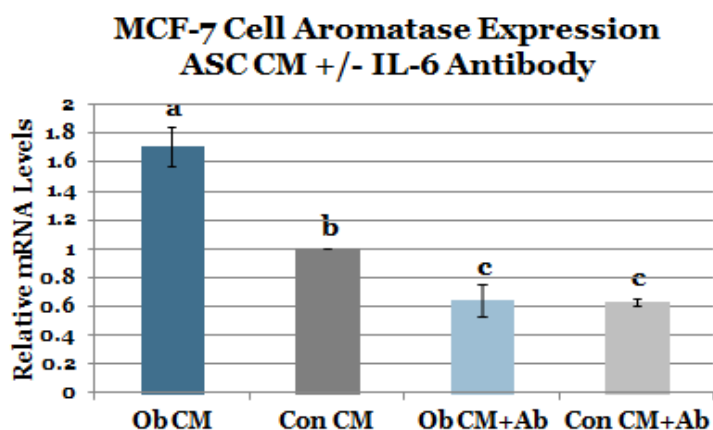
**Figure 3.** ER $\beta$  expression in MCF-7 and ZR75 cells was measured by qPCR following a 1 hour exposure to Ob or Con human sera. There were no significant differences between Ob and Con.



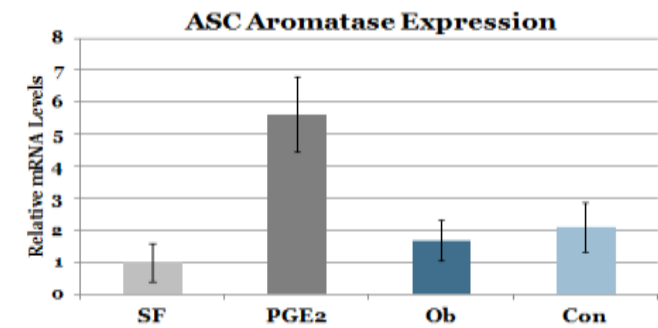
**Figure 4.** Aromatase expression was measured by qPCR in MCF-7 cells exposed to Ob or Con human serum for 24 hours, with or without the addition of the following inhibitors: LY294002 (LY, a PI3K inhibitor), Akt II inhibitor (AktII), and PD98059 (PD, a MEK inhibitor). Different letters indicate significant differences ( $p < 0.05$ ).



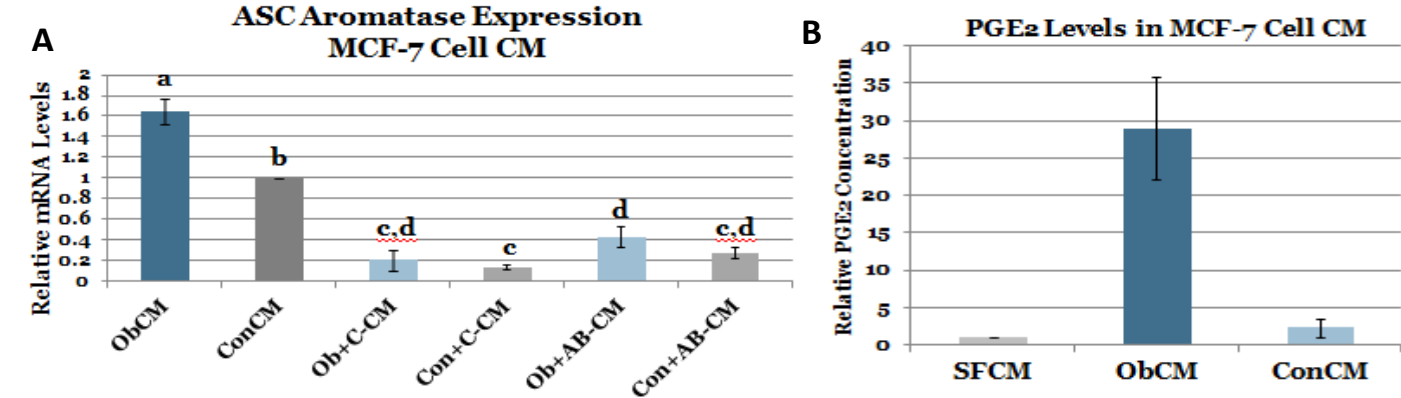
**Figure 5.** MCF-7 cell aromatase expression was measured by qPCR after a 24 hour exposure to ASC conditioned media (CM). IL-6 in the CM was neutralized by adding an IL-6 neutralizing antibody (Ab) to both the ObCM and ConCM. Different letters indicate significant differences ( $p < 0.05$ ).



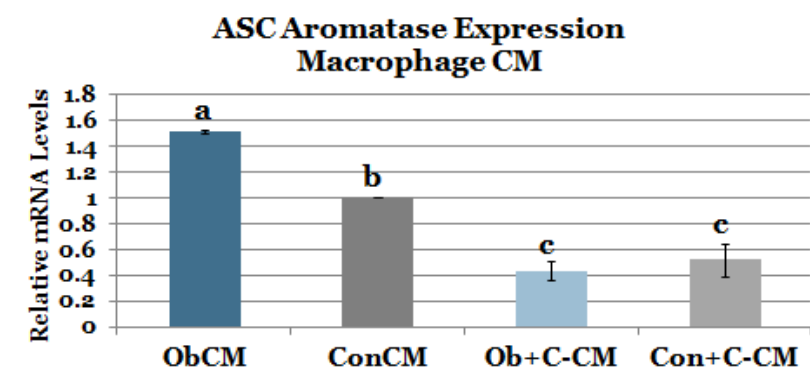
**Figure 6.** Aromatase expression in ASC exposed to Ob human serum versus Con for 24 hours was measured by qPCR. There was no significant difference between Ob and Con.



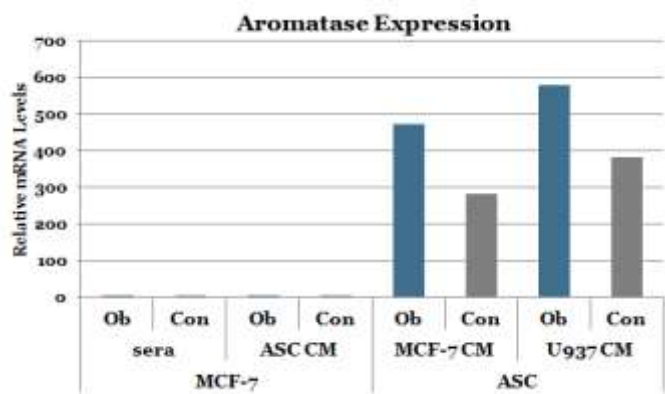
**Figure 7.** (A) Aromatase expression in ASC exposed to MCF-7 cell CM for 24 hours was measured by qPCR. Ob+C-CM and Con+C-CM indicate inhibition of COX-2 activity in the MCF-7 cells with celecoxib during the generation of CM with Ob and Con sera. Ob+AB-CM and Con+AB-CM indicate neutralization of IL-6 in both the Ob and Con sera (via the addition of an IL-6 antibody to the sera) prior to its use to generate MCF-7 cell CM. Different letters indicate significant differences ( $p<0.05$ ). (B) MCF-7 cell CM was generated as stated above, and PGE2 levels in the CM were measured by ELISA. The difference between ObCM and ConCM approached statistical significance ( $p=0.06$ ).



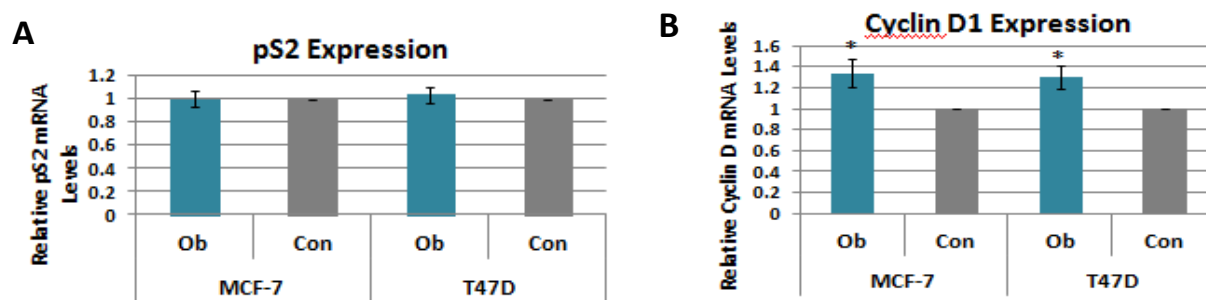
**Figure 8.** Aromatase expression in ASC exposed to U937 cell (macrophage) CM for 24 hours was measured by qPCR. Ob+C-CM and Con+C-CM indicate inhibition of COX-2 activity in the U937 cells with celecoxib during the generation of CM with Ob and Con sera. Different letters indicate significant differences ( $p<0.05$ ).



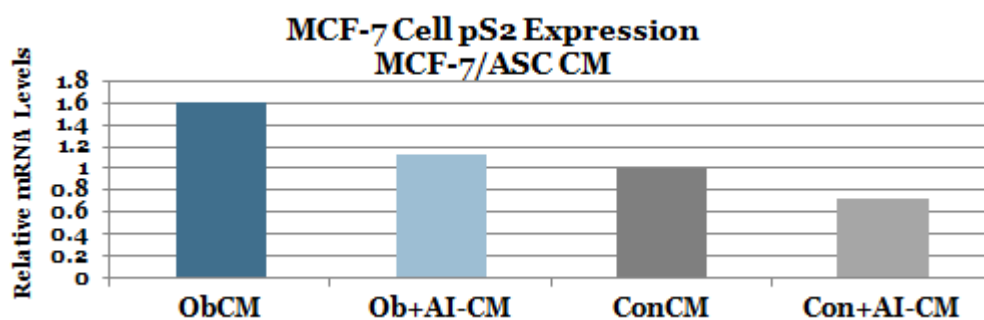
**Figure 9.** Aromatase expression in MCF-7 cells and ASC exposed to Ob or Con human sera or CM from various cell lines for 24 hours was measured by qPCR.



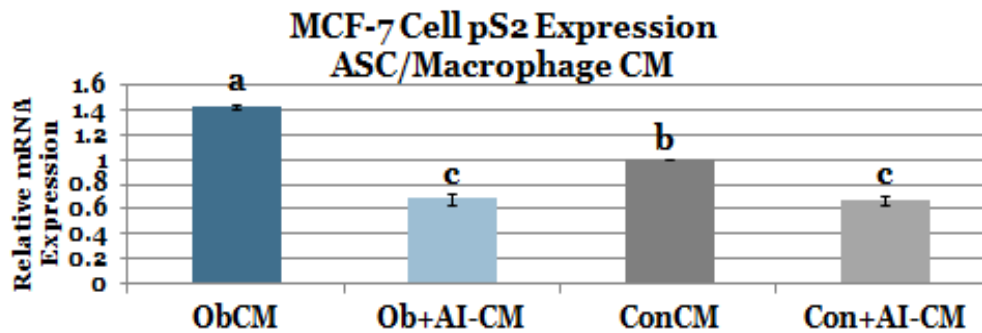
**Figure 10.** (A) ER $\alpha$  activity in MCF-7 and T47D cells exposed to Ob and Con human sera for 24 hours was measured by pS2 expression. (B) Cyclin D1 expression in MCF-7 and T47D cells exposed to Ob or Con human sera for 24 hours. Asterisks represent significant differences in comparison to Con ( $p < 0.05$ ).



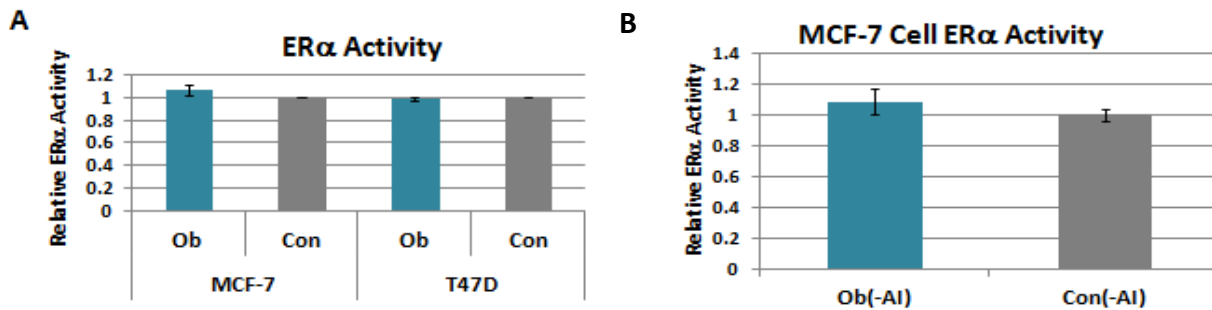
**Figure 11.** MCF-7 cell ER $\alpha$  activity, measured via qPCR analysis of pS2 expression, following a 24 hour exposure to CM from a co-culture of MCF-7 cells and ASC. This CM was generated by exposing the co-culture to Ob or Con human sera for 1 hour, removing the serum and washing the cells with PBS, then incubating the co-culture in SFM plus exogenous testosterone (ObCM and ConCM) or testosterone and the aromatase inhibitor anastrozole (Ob+AI-CM and Con+AI-CM) for 24 hours. The SFM (now CM) was then collected and used to treat separate MCF-7 cells.



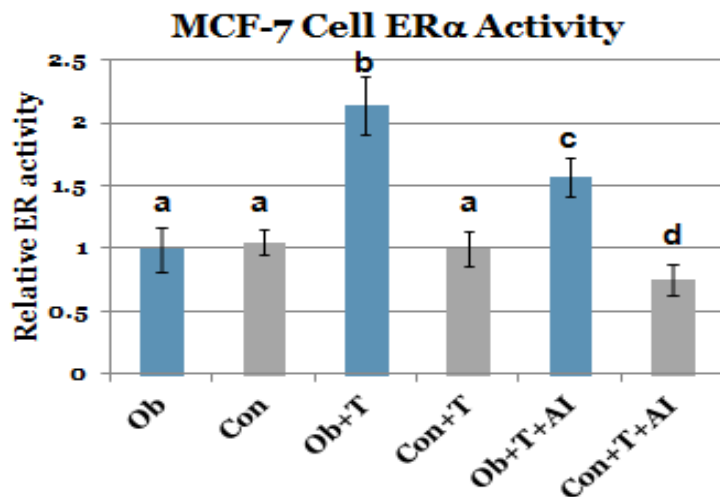
**Figure 12.** MCF-7 cell ER $\alpha$  activity, measured via qPCR analysis of pS2 expression, following a 24 hour exposure to CM from a co-culture of U937 cells (first differentiated to macrophages) and ASC. This CM was generated by exposing the co-culture to Ob or Con human sera for 1 hour, removing the serum and washing the cells with PBS, then incubating the co-culture in SFM plus exogenous testosterone (ObCM and ConCM) or testosterone and the aromatase inhibitor anastrozole (Ob+AI-CM and Con+AI-CM) for 24 hours. The SFM (now CM) was then collected and used to treat the MCF-7 cells. Different letters indicate significant differences ( $p < 0.05$ ).



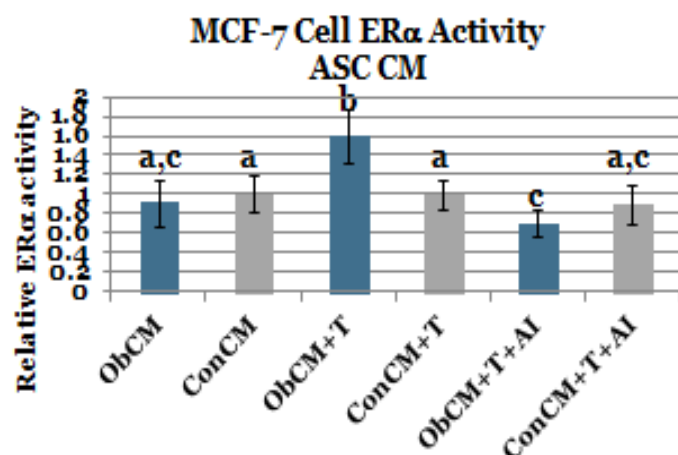
**Figure 13.** (A) ER $\alpha$  activity in MCF-7 and T47D cells exposed to Ob and Con human sera for 48 hours was measured by ERE luciferase assay. (B) ER $\alpha$  activity, as measured by ERE luciferase, in MCF-7 cells exposed to Ob or Con human sera. For this experiment, subjects that were on aromatase inhibitor treatment at the time of sera collection were eliminated from the Ob and Con sera pools (Ob(-AI) and Con(-AI)).



**Figure 14.** MCF-7 cell ER $\alpha$  activity, measured via ERE luciferase assay, following a 48 hour exposure to Ob or Con human sera alone, with exogenous testosterone added (Ob+T and Con+T), and with testosterone and the aromatase inhibitor anastrozole added (Ob+T+AI and Con+T+AI).



**Figure 15.** (A) MCF-7 cell ER $\alpha$  activity, measured via ERE luciferase assay, following a 48 hour exposure to ASC CM alone (generated with Ob or Con human sera as described previously), with exogenous testosterone added (ObCM+T and ConCM+T), and with testosterone and the aromatase inhibitor anastrozole added (ObCM+T+AI and ConCM+T+AI).



## Appendix B: Poster Abstracts

**Obesity promotes elevated aromatase expression in the breast tumor microenvironment via upregulation of prostaglandin E<sub>2</sub> production.** Bowers LW, Li R, Tekmal RR, Brenner AJ, Hursting SD, deGraffenried LA.

According to clinical correlation studies, obesity is associated with a worse breast cancer prognosis for both pre- and postmenopausal women. One mechanism for this negative effect may be endocrine therapy resistance, as obese postmenopausal patients' response rate to aromatase inhibitors is significantly lower than lean patients'. Obesity is accompanied by elevated levels of circulating cytokines and growth factors that can promote epithelial cell prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production via COX-2, and PGE<sub>2</sub> is a known regulator of adipose stromal cell (ASC) aromatase expression. Consequently, we hypothesized that exposure to obesity-associated circulating factors increases breast cancer cell production of PGE<sub>2</sub>, resulting in greater local ASC aromatase expression.

To test this hypothesis, we utilized an *in vitro* model of obesity in which we exposed MCF-7 breast cancer cells to pooled serum samples from obese (BMI  $\geq 30.0$  kg/m<sup>2</sup>) or lean (BMI 18.5-24.9 kg/m<sup>2</sup>) postmenopausal women. Following serum exposure, the cells were incubated in serum-free media for 24 hours. We then examined the effect of the resulting MCF-7 conditioned media (CM) on ASC aromatase expression, finding that obese CM produced >50% higher expression in the ASC than lean CM. Preliminary data also indicates that PGE<sub>2</sub> levels in the obese CM are approximately ten-fold greater than lean CM. Measurement of tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 6 (IL-6), and insulin-like growth factor 1 (IGF-1) concentrations in the serum samples revealed that all were higher on average in the obese versus lean subjects.

To expand on these findings, we plan to assess the role of these circulating factors as well as COX-2 signaling in the production of the breast cancer cell-mediated upregulation of ASC aromatase expression by depleting the obese serum of each factor and utilizing a COX-2 inhibitor. Estrogen production by the ASC following CM exposure will also be measured to assess its correlation with ASC aromatase expression. Through greater understanding of the signaling pathways responsible for obesity's upregulation of aromatase, we hope to

identify molecular targets that will lead to a more effective chemotherapeutic regimen for the obese postmenopausal population.

**Obesity-associated growth factor signaling promotes aromatase expression and estrogen receptor activity in breast cancer cells.** *Bowers LW, De Angel R, Brenner AJ, Tekmal RR, Hursting SD, deGraffenried LA.*

Obesity increases the risk of breast cancer by approximately 50% in postmenopausal women and is also associated with a worse prognosis. Elevated estrogen synthesis by the local mammary epithelia and adipose tissue is thought to be the principal mediator of breast tumorigenesis in this population, which primarily develops estrogen receptor alpha (ERa) positive breast cancer. However, the elevated levels of free insulin-like growth factor 1 (IGF-1) that accompany obesity are also thought to play a role. IGF-1 has significant tumorigenic effects in the breast and also regulates aromatase, the key enzyme in the conversion of androgens to estrogen. Consequently, we hypothesized that obesity increases the risk of postmenopausal breast cancer via growth factor-induced aromatase expression and/or activity in the local mammary tissue.

We have previously shown that MMTV-Wnt-1 mammary tumors from obese ovariectomized mice express higher levels of aromatase in comparison to tumors from lean ovariectomized mice. To examine the molecular pathways responsible for this effect, we have utilized an *in vitro* model of obesity in which ERa positive MCF-7 breast cancer cells were exposed to human sera obtained from postmenopausal women and pooled by BMI category. Our data indicates that exposure to obese ( $\text{BMI} \geq 30 \text{ kg/m}^2$ ) human serum stimulates greater aromatase expression in comparison to control ( $\text{BMI}: 18.5\text{-}24.9 \text{ kg/m}^2$ ). This is coupled with enhanced ERa activity when exogenous androgen is present, indicating that the increased aromatase expression results in greater estrogen production. Obese human sera also activates MCF-7 cells' Akt pathway to a greater degree than control, while MCF-7 cells expressing a constitutively active Akt demonstrate higher levels of aromatase expression in comparison to MCF-7 cells. This suggests that circulating growth factors in the obese sera, like IGF-1, may induce elevated aromatase expression via the downstream PI3K/Akt pathway.

To expand on our findings, we plan to assess whether treatment with PI3K/Akt pathway inhibitors eliminates the difference in MCF-7 cells' aromatase expression following exposure to obese versus control sera. We will also examine how inhibition of the PI3K/Akt pathway affects ERa activity in sera-exposed MCF-7 cells. Through elucidation of the signaling pathways responsible for obesity's upregulation of local aromatase expression, we ultimately hope to develop rational and effective chemopreventive and chemotherapeutic regimens for the high-risk obese postmenopausal population.